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INTRODUCTION

In both precancerous breast lesions and breast cancer, hyperproliferative activity due to oncogene activation or loss of tumor suppressor genes induces stalling and collapse of DNA replication forks, which in turn activates the replication stress response (RSR) to maintain genome integrity [1-4]. RSR is a subset of the DNA damage response that safeguards the replication process [5]; defects in RSR allow the survival and proliferation of genomically unstable cells, ultimately leading to breast cancer [4-6]. Since the initial RSR defects occur before cancer develops, RSR defects can serve as a powerful biomarker to predict the risk of cell transformation. Importantly, the presence of RSR defects distinguishes premalignant lesions and breast cancer from normal tissues, which makes these defects effective targets for both breast cancer prevention and breast cancer treatment. This project is to use cutting-edge technologies to characterize novel RSR genes and their functions in tumor suppression; identify gene signature and membrane proteins associated with defective RSR; identify drugs that target these defects; and develop RSR-defect-targeting nanoparticles for diagnostic imaging, prevention, and treatment of breast cancer. During the first three years of this project, we have validated TUSC4 as a novel RSR gene, established an RSR-defect (RSRD) gene signature, developed nanoparticles that attached to the cells expressing high level of RSRD membrane markers, and identified compound candidates that specifically targeted on RSRD cells. Here, we further investigated how TUSC4 mechanistically participates in RSR and whether TUSC4 functions as a novel tumor suppressor gene in breast cancer that suppresses mammary tumor formation in mice. In addition, we further optimized the nanoparticles that could detect RSR defect cells. Importantly, we have performed the *in vivo* experiments to validate the effectiveness of our compound candidates on RSR defect cancer cells and we are in the process of developing the nanoparticles that can carry these compound candidates for targeted therapy in the future. The progress of our fourth year research is described below.

BODY

The tasks involved in our fourth-year research include: Task 1c, Task 3b and Task 4a,b.

Task 1c. To determine whether any of these five RSR candidates functions as tumor suppressor gene in breast cancer.

As demonstrated in our previous report, among the five potential candidates, we have validated the function of TUSC4 in response to replication stress and demonstrated TUSC4 as the most promising candidate as a bona fide tumor suppressor gene in breast cancer. We have also linked the function of TUSC4 to the protein stability of BRCA1 though the mechanism was unclear. During the fourth year of this award, we have completed our studies on TUSC4 and have a manuscript submitted to Cancer Research for review.

To further support a critical role of TUSC4 in breast cancer suppression, we sought to determine whether TUSC4 expression is associated with breast cancer. We performed Western blotting analysis to measure the expression of TUSC4 in non-transformed breast cell lines, including HMEC, MCF-10A, and MCF-12A, and breast cancer cell lines with both luminal and basal subtypes (Figure 1A). We observed that TUSC4 expression was markedly higher in the non-transformed cell lines than that in breast cancer cell lines. We also evaluated TUSC4 expression on breast cancer tissue microarrays by immunohistochemical staining. As shown in the Figure 1B, TUSC4 expression was lower in the cancer tissue than that in matched adjacent normal breast tissue.

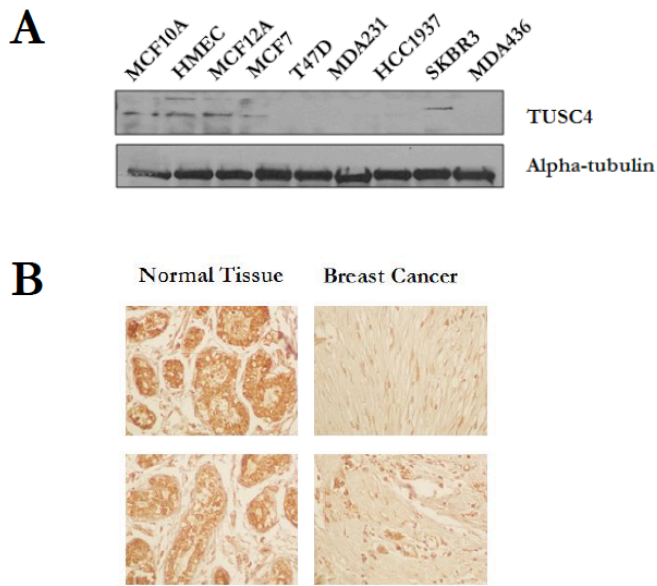


Figure 1. TUSC4 expression level is lower in breast cancer.

A. Lower TUSC4 expression levels were found in both luminal and basal types of breast cancer cell lines, while non-transformed breast cell lines (HMEC, MCF-10A and MCF-12A) exhibited higher TUSC4 expression level.

B. IHC staining indicated that normal breast tissue (left) expressed higher TUSC4 protein level than breast cancer tissues (right).

Additionally, we examined if TUSC4 defect could initiate breast tumor development in a xenograft mouse model. We injected MCF-10A cells with stable knockdown of TUSC4 expression and the control cells into the mammary fat pads of female nude mice. We closely monitored tumor formation in the mice. Notably, tumors began to form in 3 of 10 mice injected with TUSC4-knockdown cells after 3 weeks, whereas no tumors formed in the control groups (**Table 1**). These results demonstrated that loss of TUSC4 expression alone is sufficient to initiate malignant transformation of nontransformed mammary epithelial cells, which is consistent with our hypothesis that TUSC4 functions as a bona fide tumor suppressor in breast cancer.

Tumorigenicity of Orthotopically Implanted Control and TUSC4-Knockdown MCF10A cells

Number of Mice with Tumors (%)					
	Week 1	Week 3	Week 5	Week 7	Week 9
MCF10A Control	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
TUSC4-1	0/5 (0)	2/5 (40)	2/5 (40)	2/5 (40)	2/5 (40)
TUSC4-4	0/5 (0)	0/5 (0)	0/5 (0)	1/5 (20)	1/5 (20)

Table 1. 1×10^7 cells from MCF-10A control and two independent TUSC4-knockdown MCF-10A cell lines (TUSC4 #1 and TUSC4 #4) were injected per mouse into mammary fat pads of 6-week-old female nude mice. Each cell line was injected in five different mice, and tumor sizes were measured.

Our previous study demonstrated that TUSC4 is required for maintenance of the protein stability of BRCA1, which may at least in part explain how TUSC4 functions as a tumor suppressor in breast cancer. Next, we sought to determine mechanistically how TUSC4 regulates the protein stability of BRCA1. BRCA1 protein stability has been shown regulated by the E3 ligase, Herc2. We therefore investigated whether TUSC4 interacts with TUSC4 and/or BRCA1 and is functionally involved in this process. To this end, we performed immunoprecipitation with established TUSC4-overexpressing cell lines to determine the relationships among TUSC4, BRCA1, and Herc2. Reciprocally, we found that TUSC4 physically interacts with Herc2 but not with BRCA1 (Figure 2A), which strongly suggested that TUSC4 regulates BRCA1 stability via interaction with Herc2. Considering the previously reported binding functions of TUSC4 [7,8], we suspected that TUSC4 may prevent physical interaction between BRCA1 and Herc2. The binding between these two proteins [9] has been previously described, so we performed further immunoprecipitation to determine whether overexpression of TUSC4 weakens this binding. As shown in Figure 2B, endogenous Herc2 physically bound to BRCA1. Intriguingly, overexpression of TUSC4 interrupted the binding between Herc2 and BRCA1, indicating that TUSC4 may regulate BRCA1 stability by preventing physical interaction between BRCA1 and Herc2.

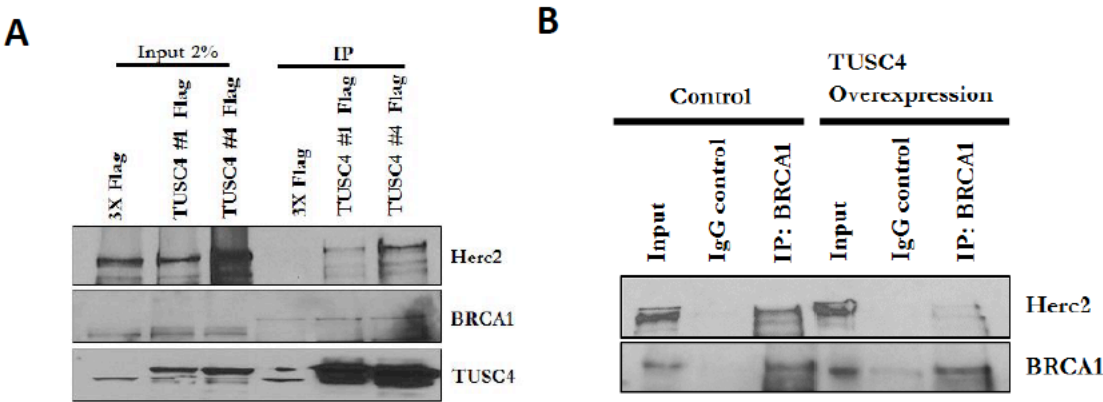


Figure 2. (A) TUSC4 physically interacts with Herc2 but not BRCA1. (B) TUSC4 overexpression reduced the binding between BRCA1 and Herc2.

Task 3b. Pilot study to test 5 top compound candidates selected from Task 3a for their effects on cancer cells.

We have previously identified MEK inhibitors (AZD6244 and CI-1040) and EGFR inhibitors (Lapatinib and Vandetanib) as the top compound candidates that can specifically target RSR defective cell lines *in vitro*. To test their therapeutic effects *in vivo*, we chose the agents that are currently used in clinic or at least under the clinical trials. Thus, we decided to test if AZD6244 (in phase III clinical trial) or Lapatinib (FDA approved) can prevent tumorigenesis caused by RSRD using a xenograft model in mice (Figure 3A). We found that both drugs were well tolerated in mice; mice didn't lose any weight or suffer ill health compared to vehicle control group. (Figure 3B). To our surprise, unlike the effect we observed from the *in vitro* MCF-10A-derived RSRD cells, Lapatinib treatment did not prevent the growth of MDA-MB-231 cells in nude mice (Figure 3C and 3D). However, AZD6244 treatment did show great effects on preventing tumorigenesis (Figure 3C and 3D). Because AZD6244 but not Lapatinib showed therapeutic potential on treating RSRD cells without noticeable side effect, we therefore only chose AZD6244 for the study proposed in Task 4b (see below). In addition, we also identified ERK inhibitor (SCH772984, phase I clinical trial) that can effectively target RSRD cells *in vitro*. Therefore, in the following year, we will further test the efficacy of SCH772984 *in vivo*.

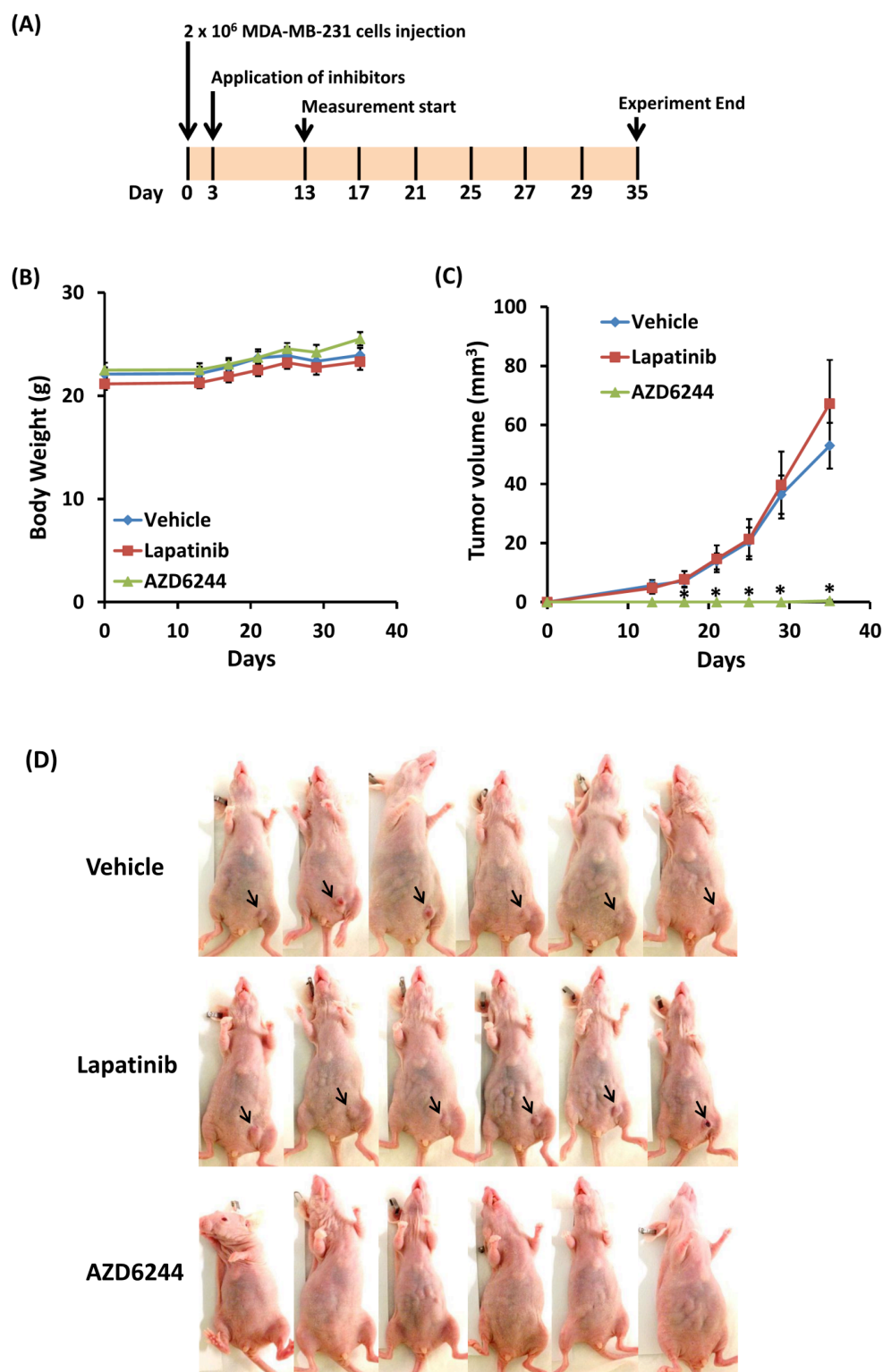


Figure 3 MEK inhibitor (AZD6244) but not EGFR inhibitor (Lapatinib) effectively prevents tumorigenesis *in vivo*. (A) The experimental design for assessing drug efficacy *in vivo* (B) The mean body weight (\pm SEM) illustrated based on gram ($n = 10$) (C) The mean tumor volume (\pm SEM) illustrated based on mm³ ($n = 10$). * $P < 0.05$ compared with vehicle treatment (two-tailed t -test). (D) Six mice from each treatment group were used to represent the drug efficacy.

Task 4a. To develop nano-imaging technology to detect RSR-defective breast cancer cells through binding of nano-imaging particles to the RSR-defect-specific membrane proteins.

In our previous progress report, we have shown our success in developing antibody-conjugated hollow gold nanoparticle (HAuNS) to target RSRD cells. In the fourth year of this award, we further optimized these nanoparticles and reduced their size in order to improve tumor targeting efficiency. The average diameter of HAuNS was reduced from 136.6 nm to 52.7 nm (~85% reduced in total volume) (Figure 4). The homogeneity of HAuNS was also improved (polydispersity from 0.293 to 0.005) (Figure 4).

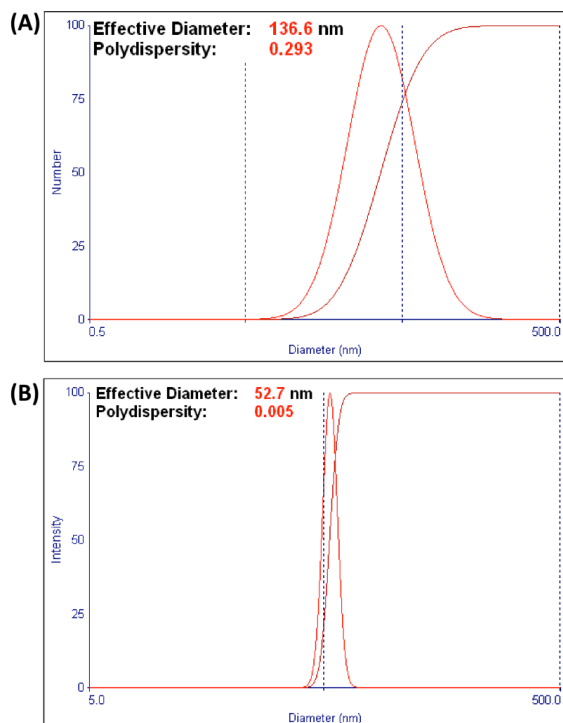


Figure 4. Optimization of HAuNS. (A) The results of raman spectroscopy show particle size and homogeneity of HAuNS reported previously (B) The results of raman spectroscopy show particle size and homogeneity of HAuNS after optimization.

We then tested again and see if the optimized HAuNS after conjugated with the antibodies against AXL and Jag1, two RSRD membrane markers that we identified, could still specifically attach RSR-defect breast cancer cells. We incubated HAuNS-AXL, HAuNS-Jag1, and HAuNS-GIgG with RSR-intact T47D breast cancer cells and two RSR-defect (MDA-MB-231 and Hs578T) breast cancer cell lines. After incubation, the cells were stained with DAPI and the dark field images were obtained. The results of dark field images showed that both HAuNS-AXL and HAuNS-Jag1 were retained only on the surface of RSR-defect breast cancer cell lines (Figure 5). These results indicated that our optimization process was successful and we have generated HAuNS with much smaller size but remained their capacity to be conjugated to the specific antibodies for detecting the RSRD breast cancer cells. We planned to test these nanoparticles in mice. However, our collaborator Dr. Chun Li's lab moved from the north campus to the south campus in May. It took two months for him to set up his new lab, and it took even longer to resume any animal work. Therefore, the planned *in vivo* experiments were delayed and we will resume this part of study in the end of this month.

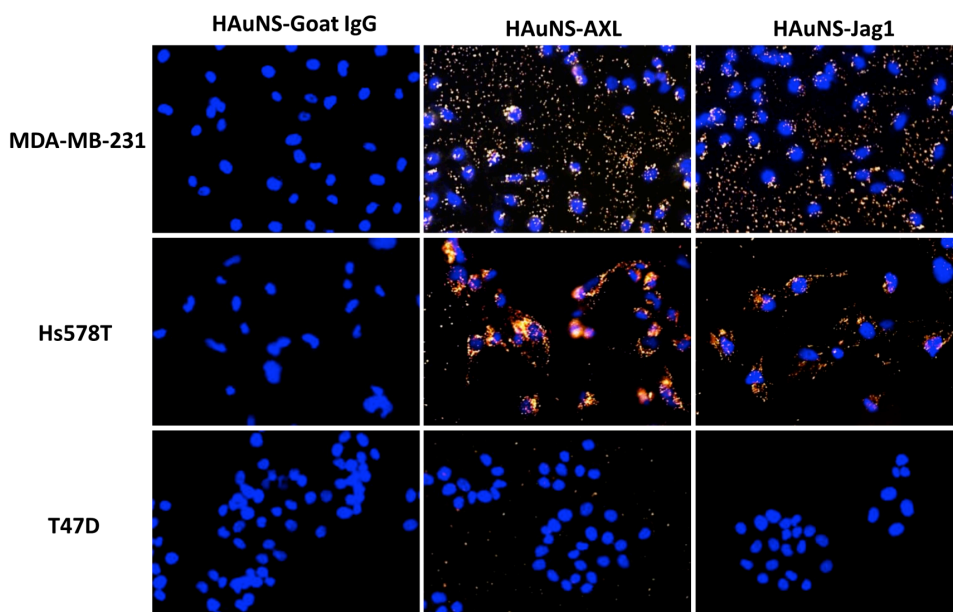


Figure 5. New antibody-conjugated HAuNS remains the specificity and efficiency on targeting RSR defective breast cancer cells. Two RSR defective breast cancer cell lines, MDA-MB-231 and Hs578T, and RSR intact breast cancer cell line, T47D, were used to test specificity of new antibody-conjugated HAuNS. HAuNS-Goat IgG is used as a negative control. Scale bar is 50 μm .

Task 4b. To develop nanoparticles to kill RSR-defective breast cancer cells through their binding to the RSR-defect-specific membrane proteins on cancer cells.

We chose AZD6244 as the drug candidate (Task 3b) to be co-conjugated with AXL or Jag1 antibody to the optimized HAuNS (Task 4a). We first modified the alcohol functional group of AZD6244 to thiol functional group so it can be conjugated with HAuNS through disulfide bond (Figure 6). This chemical modification was very time consuming because of three major reasons: (1) The alcohol to thiol reaction is not commonly performed so the available references are quite limited (2) The scale of a typical chemical reaction is usually performed in a relatively large scale. In our project, we had to reduce the scale and that unavoidably affected the yield rate (3) Due to the similarity of chemical properties, it required a long process for the compound purification. We have actively sought advices from organic chemists to resolve all these difficulties. The modified AZD6244 will be co-conjugated with either AXL or Jag1 antibody onto the HAuNS for future *in vitro* and *in vivo* studies.

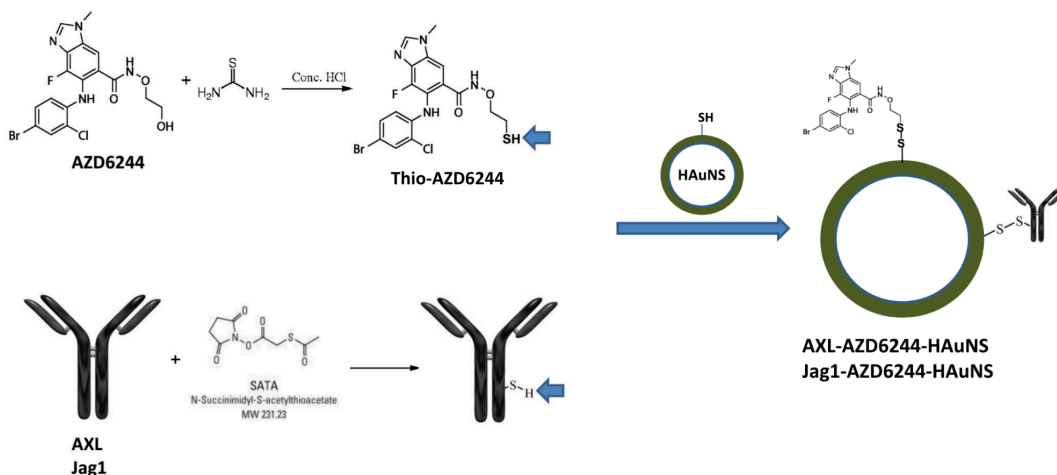


Figure 6. The procedure of drug, antibody, and H AuNS conjugation. The alcohol function group was modified to thiol functional group. The reaction was adopted base on previous study [Buter and Kellogg, Organic Syntheses, Collective Volume 8, 592, (1993)]. Thio-AZD6244 and antibody will be conjugated with H AuNS to generate either AXL-AZD6244-H AuNS or Jag1-AZD6244-H AuNS.

KEY RESEARCH ACCOMPLISHMENTS

- (1) We demonstrated that the expression of TUSC4 was reduced in breast cancer cell lines and breast cancer tissues compared to normal breast cell lines and normal breast tissue.
- (2) We demonstrated that TUSC4 defect alone was sufficient to transform normal breast epithelial MCF-10A cells and form tumors in mice.
- (3) We found that TUSC4 binds Herc2 and interferes its binding to BRCA1, and, consequently, protects BRCA1 protein from polyubiquitination and degradation.
- (4) We have successfully validated the targeting effect of MEK inhibitor, AZD6244 on RSRD breast cancer cells in a xenograft mouse model.
- (5) We have successfully optimized and reduced the size of hollow gold nanoparticles in order to improve their targeting efficiency *in vivo*.
- (6) We have modified the alcohol functional group of AZD6244 to thiol so it can be conjugated to H AuNS.

REPORTABLE OUTCOMES

The progress of this project in the past year has led to one paper published in Nature Communications [10], and two manuscripts under review in Science and Cancer Research, respectively. Our intriguing findings have also allowed me to be invited for presentation at the 3rd World Congress on Cancer Science & Therapy at San Francisco, and two poster presentations by my postdoctoral fellow and my Ph.D. student at AACR annual meetings at San Diego [11,12].

CONCLUSION

During the fourth year of this project, we have made significant progress in several of our proposed tasks. We found that TUSC4 is a potent tumor suppressor gene in breast cancer; its deficiency alone can sufficiently transform normal breast epithelial cells and promote tumor growth in mice. We further demonstrated that mechanistically TUSC4 protects BRCA1 protein from degradation by interfering its binding to Herc2 E3 ligase.

In addition, we have validated the *in vivo* effects of MEK inhibitor, AZD6244 on targeting RSRD breast cancer cells in a xenograft mouse model.

Finally, we have successfully optimized and reduced the size of H AuNS and modified a functional group of AZD6244 so it can be conjugated to H AuNS.

REFERENCES

1. Bartkova J, Horejsí Z, Koed K, Krämer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, Ørntoft T, Lukas J, Bartek J. (2005) DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature*. 434:864-870.
2. Bartkova J, Rezaei N, Liontos M, KaTUSC4aidos P, Kletsas D, Issaeva N. et al., (2006) Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* 444:633-637.

3. Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P et al., (2006) Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* 444: 638-642.
4. Halazonetis TD, Gorgoulis VG, Bartek J. (2008) An oncogene-induced DNA damage model for cancer development. *Science* 319:1352-1355.
5. Osborn AJ, Elledge SJ, Zou L. (2006) Checking on the fork: the DNA-replication stress-response pathway. *Trends Cell Biol.* 12(11):509-516.
6. Dapic V, Carvalho MA, Monteiro AN. (2005) Breast cancer susceptibility and the DNA damage response. *Cancer Control* 12:127-136.
7. K, Kawashima H, Ohtani S, Deng WG, Ravoori M, Bankson J, et al. (2006) The 3p21.3 tumor suppressor NPRL2 plays an important role in cisplatin-induced resistance in human non-small-cell lung cancer cells. *Cancer Res*; 66, 9682-90.
8. Jayachandran G, Ueda K, Wang B, Roth JA, Ji L. et al. (2010) NPRL2 sensitizes human non-small cell lung cancer (NSCLC) cells to cisplatin treatment by regulating key components in the DNA repair pathway. *PLoS One*; 5; 8, e11994.
9. Wu W, Sato K, Koike A, Nishikawa H, Koizumi H, Venkitaraman AR, Ohta T. (2010) Herc2 is an E3 ligase that Targets BRCA1 for degradation. *Cancer Res* ; 70, 6348-92.
10. Peng G, Chun-Jen Lin C, Mo W, Dai H, Park YY, Kim SM, Peng Y, Mo Q, Siwko S, Hu R, Lee JS, Hennessy B, Hanash S, Mills GB, Lin SY. (2014) Genome-wide transcriptome profiling of homologous recombination DNA repair. *Nat Commun* 5:3361.
11. Lin CC, Lin S-Y.. The replication stress response defect is associated with tumor-initiating cell formation, AACR, San Diego, CA, 4/2014.
12. Peng Y, Lin S-Y.. TUSC4 functions as tumor suppressor by regulating BRCA1 stability and functions, AACR, San Diego, CA, 4/2014.